RELATIVE REQUIREMENTS OF CHLORINE AND OF CHLORINE DIOXIDE FOR THE DENATURATION OF A PROTEIN

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RELATIVE REQUIREMENTS OF CHLORINE AND OF CHLORINE DIOXIDE FOR THE DENATURATION OF A PROTEIN

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INTRODUCTION

THE RELATIVE REQUIREMENTS OF CHLORINE AND OF CHLORINE DIOXIDE FOR THE DENATURATION OF A PROTEIN

CHAPTER I

INTRODUCTION

The initial objective of this investigation was the determination of the relative requirements of chlorine and chlorine dioxide for the denaturation of a protein. It has been planned to use this information to evaluate the possible role of denaturation of the proteins of bacteria in the bactericidal attack upon the organisms in water solutions.

In order to determine the relative effects of the two gases, it was first necessary to determine a suitable technique for assaying the degree of denaturation or changes which take place when aqueous solutions of the two gases are added to solutions of crystalline egg or bovine plasma albumins. Two dye indicators were considered on the assumption that any ensuing color change due to denaturation could be detected with a colorimetric apparatus. It was found, however, that this method was not suitable for the study at hand. The method subsequently used consisted of the measurement of change in optical density or light adsorption using a colorimeter with a 150 mm light path and a suitable color filter. This method permits the estimation of a change in optical density caused by the changes in particle size or number, but it also determines any color produced in the particles or solution. Because of this weakness it

was necessary to use another colorimeter in which the separation of turbidity changes from color changes were made possible by shifting the position of the sample in the light path. The validity of the techniques was to be tested by comparison of the changes produced by increasing temperature on the same protein, utilizing the same instruments.

It must be borne in mind that the experiments were conducted at a pH of 7.8, using sodium bicarbonate as a buffer for both the albumin and the chlorine and chlorine dioxide solutions. Proteins, and albumins in particular, are most sensitive to coagulation or denaturation effect at a pH near the iso-electric point, or at pH 4.6 to pH 4.9. It must also be remembered that chlorine is most effective bactericidally at a pH which is on the acid rather than on the alkaline side. Conditions under which the experiments were conducted were therefore not the most favorable for coagulation phenomena to occur. On the other hand, chlorine or chlorine dioxide as used in water disinfection practice is used in the pH range of the experiments (pH 7.8).

It is hoped that the information derived from these experiments will aid in the understanding of the possible mechanism involved in the bactericidal qualities of the two gases.

CHAPTER II

THEORETICAL DISCUSSION

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Although the bactericidal activity of chlorine and of chlorine dioxide has been established, the actual mechanisms involved are not too well understood, and are still open to conjecture. Various hypotheses have been postulated over the years, ever since the infancy of chlorination practice. It was thought earlier that chlorine kill was due to an oxidative process, and that the chlorine reacted directly with water to produce nascent oxygen which had a definite bactericidal effect on the cell. This theory has been since discarded since it has been shown that bactericidal effect is not proportional to the concentration of nascent oxygen produced.[1] The theory is further disproved by the fact that hydrogen peroxide, which releases nascent oxygen readily, is ineffective as a bactericidal agent. Another theory states that the reaction is purely physicochemical in nature, and states as follows:[2] "The cell wall structure and the cell contents contain certain proteins and amino groups with which the chlorine reacts. This action alters the chemical characteristics of the cell contents and destroys life, or may cause disintegration of the cell structure." The present study has been designed in an attempt to find an answer to this theory. Will chlorine and chlorine dioxide denature egg albumin? Will this denaturation reaction require the relative amounts of chlorine and chlorine dioxide needed for a bactericidal effect?

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[1] Water Quality and Treatment, (New York: The American Water Works Association, Incorporated, 1950), p. 209.

[2] Ibid., p. 209

It is interesting to note that early reference is made to the physiological significance of denaturation, and the suggestion of the possibility that "denaturation and its reversal are biological reactions which may be of importance in ordinary cellular processes."[3]

The work of Green and Stumpf[4] has offered still another explanation for the possible mode of action of chlorine and of chlorine compounds upon bacterial cells. Their investigations were based on the proposition that since chlorine is effective at the trace level, (0.2 to 2.0 ppm, in water free of organic material), the implication is that it must inhibit a key enzymatic process. By using techniques involving the physiological chemistry of bacterial cells, they found this critical process to be the oxidation of glucose by the bacterial cell, and they showed further, that once the power of glucose oxidation is lost, bacterial cells die. Two statements made in the course of their paper are important and pertinent to the concept of the bactericidal mechanism. One states that "Proteins in general react non-specifically with chlorine and therefore reduce the amount available for reaction with enzymes." This would seem to minimize the theory of direct protein reaction, but a more accurate definition of "non-specific" protein reaction would have to be given by the writers in order to interpret this statement correctly.

The other statement which is of interest is that "when sulfhydryl groups become oxidized by chlorine, enzymatic activity is abolished

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[3] M. Bodansky, Introduction to Physiological Chemistry, (New York: John Wiley and Sons, Incorporated, 1934), p. 118.

[4] D. E. Green, and P. K. Stumpf, Jour. A. W. W. A., 38, 1301-5 (1946).

irreversably". If this is true, the question then arises as to whether or not chlorine or chlorine dioxide oxidizes the sulfhydryl groups of certain amino acids as well, cysteine for example, and whether or not this has any effect on the overall structure of the cell or the protein.

The possibility of denaturation of the protoplasmic ingredients, i.e., the proteins in the cell, should therefore also be considered, and it is with this in mind that the work reported in this paper was prosecuted. It must be realized, of course, that with our present source of knowledge it is impossible to duplicate a protoplasmic solution in vitro. It is possible, however, to take a typical protein, such as egg albumin, and by using relatively small protein concentrations with small concentrations of chlorine or chlorine dioxide, to make observations on the changes brought about by these two gases.

The concept of denaturation, like the concept of the mechanism of bactericidal effect of chlorine compounds, has undergone considerable change over the years, and is still in a state of flux. While a precise definition of denaturation is lacking, and while the change from the fresh or "native" (undenatured) form to the denatured protein is not clearly understood, it is agreed that denaturation is a process peculiar to proteins. Bull[5] states that "Denaturation is a characteristic but ill defined series of changes which many proteins undergo when subjected to a number of relatively mild physical and chemical agents". These agents include heat, strong acids and bases, alcohol, alkyl sulfates, urea, high

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[5] M. Sahyun, et al., Outline of the Amino Acids and Proteins, (New York: Reinhold Publishing Corporation, 1948), p. 77

pressures, surface forces, and ultraviolet light, as well as salts such as lithium thiocyanate. Proteins exhibit a number of changes in the denatured state, mainly: (1) decreased solubility, (2) increased digestibility by proteolytic enzymes, (3) exposure of oxidizing and reducing groups, notably the sulfhydryl groups, (4) loss of enzymatic properties if the protein is an enzyme, (5) modification of antigenic properties, and (6) decreases of the diffusion constant and increase of the viscosity of the protein solution. However, these changes may not all always be present in the denatured protein.

The heat coagulation of egg white is a familiar example of denaturation phenomena. It is said to involve three distinct processes:[6] denaturation, flocculation, and irreversable coagulation. The denaturation is due to some modification in the structure of the protein. The flocculation is a result of that change, and may be recognized by the appearance of the coagulated material. The floc is said to be irreversably coagulated when it is no longer peptized or dissolved by either acid or base. By analogy, if this sequence of reaction was to be followed by the two gases used in the experiments, then the chlorine or chlorine dioxide would be the denaturants, the flocculation would be induced by mechanical mixing, and the coagulation, if it occurred, would be detected either visibly or by turbidimetric methods.

The pH at which the reaction takes place is a critical factor. If an albumin solution is heated at a pH considerably removed from the

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6 H. B. Weiser, A Textbook of Colloid Chemistry, (New York: John Wiley and Sons, Incorporated, 1950), p. 304.

isoelectric point, there may not be any visible evidence of denaturation. If the sample is then brought to the isoelectric point (pH 4.6 to 4.9), the sample is observed to coagulate, indicating that some change or denaturation has occurred. This is an important criterion of denaturation phenomena, and it is used later in one of the experiments.

Various theories have been proposed as the actual mechanism of the denaturation process. Wu[7] suggested that the change of the native protein to the denatured form could result from the unfolding of the closely folded peptide chains, or a change from a unique and highly specific structure of the native to the much more random arrangement of the denatured form.

It was suggested by Hendrix[8] earlier that denaturation as well as coagulation may involve the condensation of opposite groups of adjacent molecules.

A more recent theory has been proposed by Haurowitz[9]. He states, "There is no doubt that the structure of the native proteins, the tight folding of their peptide chains, is maintained primarily by salt linkages between the positively charged groups of arginine and lysine on the one hand, and by the negatively charged carboxyl groups of aspartic and glutamic acid on the other." The denaturation of the protein is the direct result of the cleavage of these salt bridges. He uses this concept to

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[7] H. Wu, Chinese J. Physiol., 5, 321, (1931)

[8] B. M. Hendrix and V. Wilson, J. Biol. Chem., 79, 389, (1928).

[9] F. Haurowitz, Progress In Biochemistry, (New York: Interscience Publishers, Incorporated, 1950), p. 127.

explain the denaturing action of acids and alkalies. The negatively charged COO^- groups are converted into the uncharged COOH groups by acid, and the positively charged NH_3^+ groups are converted into uncharged NH_2 groups by alkali. Heat denaturation is explained on the same basis, the salt bonds being ruptured by the thermal motion of the peptide chains.

In studying the phenomena of denaturation of proteins, the fact that certain chemical agents act as dispersants must also be considered in an analysis of the overall problem. It is known, for example, that urea acts as a dispersant above a certain concentration, and that some of the cyanates may also act as dispersants.[10] This could result in a sort of "masking effect", with denaturation occurring at first, and with the floc or coagulate being subsequently dispersed by the same agent.

If we assume for the moment that the Haurowitz conception of denaturation is the correct one, and if we draw a hypothetical structure of the albumin molecule, it might be interesting to see how the chlorine or chlorine dioxide could attack this structure, and at what points the attack would most likely be.

A structural diagram of the protein, together with some of the amino acid radicals, follows on the next page.

In order to follow the Haurowitz hypothesis, the chlorine could be expected to attack the amine (NH_2) radical and thereby rupture the salt bridge. The fact that chlorine compounds do form chloramines, with ammonium chloride, (NH_4Cl), for example, would lead one to this assumption.

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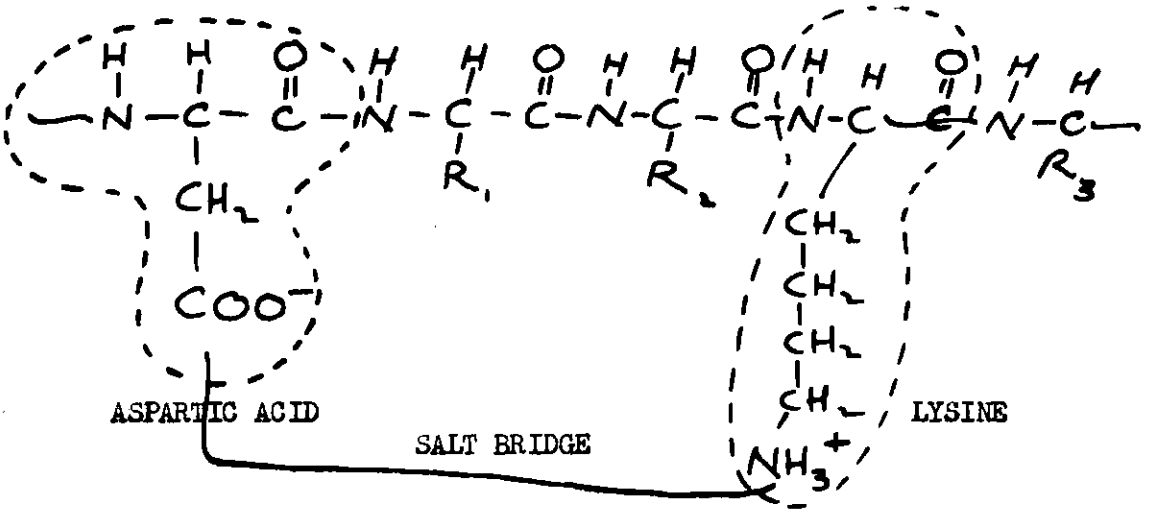
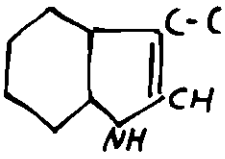
[10] S. B. Holtham and F. Schutz, Biochemica et Physica Acta, 3, 65 (1949).



R_1 = HO-C$_6$H$_4$-CH$_2$-CH(NH$_2$)-COOH TYROSINE

R_2 = C-CH$_2$-CH(NH$_2$)-COOH TRYPTOPHANE



R_3 = SH-CH$_2$-CH(NH$_2$)-COOH CYSTEINE

The ease with which aromatic acids such as tyrosine and tryptophane can be oxidized must be considered in a study of the interaction of chlorine and of chlorine dioxide with the proteins containing these acids. The oxidation of the cysteine amino acids, particularly the (SH) or sulfhydryl group of the acid, is another possibility.

One would also expect some substitution of the chlorine on the nitrogen of the peptide linkage, $-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-$, and that this peptide oxidation might interfere or compete with chloramine formation in the lysine epsilon radical. Although it is not known definitely, it is surmised that the sulfhydryl group of many enzymes comprises the haptophor radical. This hypothesis is supported by the known formula and behaviour of the antibiotics, penicillin and gramicidin.

CHAPTER III

EXPERIMENTAL

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A. Apparatus

1. Apparatus Used for Preparation of Chlorine and Chlorine Dioxide Solutions

An all-glass apparatus used for the preparation of chlorine and chlorine dioxide solutions in the experiments was designed originally by Dr. Robert S. Ingols for use in the sanitary chemistry laboratory. It consists of a removable 100 ml dropping funnel fitted by standard taper joints to a spherical shaped flask for the reaction mixture. A small piece of glass tubing leads from the outside of the flask well to the bottom of the flask to give a steady mixing of the contents by the intake of air under vacuum into the reaction mixture of all times. A glass tubing leads through a ball and socket joint from the reaction flask to a 250 ml wash bottle, and then to a second 250 ml wash bottle for the final solution of the chlorine or the chlorine dioxide. The second bottle is attached by rubber tubing to a vacuum source.

2. Colorimeters

Two colorimeters were used in the experiments. One of these, the Lumetron Colorimeter Model 450, uses a light path up to 150 mm long in low form 100 ml Nessler tubes. Readings can be made on a dial in terms of either optical density or per cent transmission. The instrument is equipped with suitable color filters for different color ranges. Because

of its long light path it was designated to give maximum sensitivity for the low concentrations of matter normally found in water.

The other colorimeter used is the Lumetron Photocolorimeter Model 402-E. This is a versatile instrument which is equipped with cuvettes of varying widths and volumes as suitable. The cuvette used in the experiments has a length of 25 mm and a volume of 28 ml. The colorimeter is equipped with a set of monochromatic filters as well as the ordinary color filters. The red monochromatic filter was used in all experiments. The Model 402-E makes it possible to note changes in turbidity as well as color changes. This differentiation is made by taking two readings, one with the sample cuvette directly against the photocell, and the other with the cuvette moved a certain distance away from it. The total distance across the compartment is 170 mm. A difference in the two readings is due to the light scattering by the particles in the solution and gives a direct measurement of the turbidity of the sample.

3. Centrifuge

All centrifuging in the experiments was done with an International Centrifuge, Size 1, Type C, at a speed of 1600 rpm, and using the attachment with four 250 ml bottles, having an arm length of 9 cm.

4. Mechanical Mixer

All mechanical mixing in the experiments was done with a low speed multiplace mixer, with a 1/50 H.P. motor.

5. De-ionizing Column

A mixed bed synthetic zeolite resin in a cylindrical column was used to de-ionize all distilled water used in the experiments.

B. Materials

1. Calcium Hypochlorite and Sodium Chlorite

The calcium hypochlorite used in the preparation of the chlorine solutions was Baker's U. S. P. grade, and the sodium chlorite used in the preparation of the chlorine dioxide was a sample of commercial grade obtained from the Mathieson Chemical Company, New York City.

2. Crystalline Egg Albumin

The crystalline egg albumin was purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. No further crystallization or purification was attempted. Although it is possible to recrystallize the egg albumin with concentrated ammonium sulfate, this procedure was not followed since it involves a prolonged period of dialysis in distilled water for the removal of all ionic material. To keep ionic contamination at a minimum, and also to reduce the possibility of bacterial contamination, all albumin solutions were prepared fresh for every experiment.

3. Bovine Plasma Albumin

The bovine albumin was purchased from Armour Laboratories, Chicago, Illinois. No further purification was attempted for the same reasons stated above. This material was much more expensive than the egg albumin and was used sparingly.

4. Dye Indicators

Two dye indicators were used in the early part of the experiments. Sodium nitroprusside dye was purchased from the Southern Scientific Company, Atlanta, Georgia. The Orange I dye was obtained from the Georgia Institute of Technology Experiment Station.

5. De-ionized Water

All water used in the preparation of the chlorine and chlorine dioxide solutions and in the preparation of the albumin solutions was distilled and de-ionized through a zeolite exchanger to eliminate or minimize the effect of any ionic material, especially ammonia.

6. Buffer

All solutions were buffered with sodium bicarbonate, Baker's C.P., to a pH of 7.8, and checked with a Beckman pH meter.

7. Acid Reagent

Sulfuric acid used in the preparation of chlorine and chlorine dioxide, and acetic acid used in some of the pH adjustments, was of C.P. grade, manufactured by Du Pont.

8. Titration Reagents and Chemicals

Titration chemicals used in the calibration of chlorine and chlorine dioxide solutions were as follows:

a. Sodium thiosulfate - Baker's C.P.

b. Potassium iodide - Merck, reagent grade.

c. Potassium dichromate - Baker's C.P.

d. Starch solution - Baker's C.P. 5.0 gm per leter of water.

9. Amino Acids

Amino acids used in one of the experiments were manufactured by Eastman Kodak, Rochester, New York.

10. Filtration

Filtration where used in the experiments was done through Corning Fiberglass glass wool in a Buchner type funnel.

C. Procedure and Results.

1. Preparation of Chlorine and Chlorine Dioxide Solutions

The method of preparation follows that as described by Ingols and Ridenour.[11] Ten ml of concentrated sulfuric acid is dissolved in 90 ml of de-ionized water and poured into the dropping funnel with the stop clock closed. Five grams of calcium hypochlorite in the case of the chlorine or five grams of sodium chlorite in the case of the chlorine dioxide, are dissolved in 100 ml of de-ionized water and poured slowly into the reaction flask. The wash bottle and the final solution bottle are both about half filled with de-ionized water and the glass tubing and rubber tubing connected for vacuum since air must be drawn continuously through the system. The amount of water in the final solution bottle will vary and will depend on the final concentration desired. Since only small concentrations were used, this volume was not at all critical. The vacuum was then turned on, and the system checked for leaks, and to see if there was constant bubbling in all of the flasks. The stop cock was then opened, and the acid allowed to

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[11] R. S. Ingols and G. M. Ridenour, Jour. A. W. W. A., 40, 1207-26 (1948).

enter dropwise into the reaction bottle. The reaction was considered completed when all the acid was used up. The chlorine solutions were carefully checked for development of any yellow tint or color which would indicate contamination by chlorine dioxide. In any case where this was found the solution was discarded. Final solutions were then placed in glass stoppered bottles and placed in the refrigerator. Solutions were not used which had been standing for more than two weeks.

2. Standardization of Chlorine and Chlorine Dioxide Solutions

The chlorine and chlorine dioxide solutions were standardized or calibrated to the strength desired, usually 100 ppm in terms of mg per liter, by titration with .01 Normal sodium thiosulfate, potassium iodide, and using starch as an end point indicator. Preparation of thiosulfate and the titration procedure was essentially that as described in Standard Methods[12]. Titration of chlorine solutions was carried on in slightly acid medium with a few drops of sulfuric acid, and for chlorine dioxide solutions in a slightly alkaline medium with 25 mg of sodium bicarbonate. Calibrated solutions were adjusted to pH 7.8 with sodium bicarbonate and kept in glass stoppered bottles in the cold room or refrigerator at all times. A final check as to the strength of each solution was made prior to each experiment.

3. Preparation of Albumin Solutions

Albumin solutions were prepared fresh to eliminate the possibility of bacterial contamination. The albumin, both egg and the bovine

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[12] American Public Health Association and the American Water Works Association, Standard Methods for the Examination of Water and Sewage, (New York: American Public Health Association, 1946), p. 99.

type, was weighed on a chainomatic type balance, and was generally either 500 or 100 mg per liter. The albumin was then placed on a watch glass and made into a thin paste using a stirring rod with a rubber policeman and a few drops of water. This was then dissolved in a liter of water and stirred slowly. The pH was then adjusted to 7.8 with sodium bicarbonate. The solution was then poured into four 250 ml centrifuge bottles and centrifuged at 1600 rpm for ten minutes in most cases. It was sometimes necessary to filter the egg albumin after centrifugation through fine glass wool in a Büchner funnel in order to remove some of the heavier particles and in order to make a more homogeneous solution. It should be mentioned here that there was some difference in the properties of the egg and the bovine albumin solutions. The egg albumin did not seem to go into solution completely but formed rather a type of colloidal suspension. The bovine albumin, on the other hand seemed to go into solution almost completely, and showed a very low turbidity even at 500 ppm.

4. Heat Effect Upon Egg Albumin With Sodium Nitroprusside Dye

The sodium nitroprusside dye has been used or reffered to in the literature as an indicator of denatured or heat coagulated albumin[13]. The method as it has been used previously is to place the albumin in a slightly ammoniacal solution of the dye. If the albumin has been denatured or coagulated a purple color should develop. Since purely visual color changes are sometimes rather difficult to detect it was hoped that this method could be used with a colorimeter, and that it

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[13] F. G. Hopkins, Nature, 126, 328 (1930).

could later be used for detection of denaturation or coagulation of egg albumin by chlorine and chlorine dioxide. The method was tested using heat as a denaturant.

One liter of dye solution was prepared with approximately 100 mg per liter. 100 mg of crystalline egg albumin was then added and the solution filtered. The solution was then adjusted to pH 7.8 with a dilute ammonium hydroxide, since the dye works only in the presence of this reagent. 200 ml of this solution was then pipetted into each of four beakers. One beaker was left at room temperature and the other three heated to temperatures of 40, 60, and 80° Centrigrade respectively to induce denaturation. Beakers were removed a few seconds after the desired temperature was reached, and cooled to room temperature. 100 ml out of each beaker was then carefully poured into four Nessler tubes, and the heights of each adjusted to 150 mm. Optical density readings were taken on the Colorimeter Model 450 using all of the available color filters.

The data as shown in Table I and Figure 1 indicates that while some rise in optical density has occurred the pattern of the rise is the same with every filter.

5. Effect of pH and of Chlorine Upon Optical Density of Orange I Dye

Reference is made in the literature to the use of Orange I dye as an indicator of denaturation of a protein[14]. It is based on the proposi-

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[14] B. Carroll, Science, 111, 387-8 (1950).

tion that native proteins have an affinity for anionic dyes, and that this affinity is lost in the presence of denaturants, such as sodium hydroxide or heat. It was thought that this method could be applied to chlorine and chlorine dioxide denaturation, and the ensuing color change noted with a colorimeter.

In the experiments which follow, however, it was found that both small pH changes within the range of 7 to 8 and the chlorine itself affect the optical density of the dye. For checking the pH effect, a 1×10^{-6} molar solution of the dye was prepared in two steps, by dissolving 328 mg (one millimolecular weight) of dye in a liter, and then by diluting 1 ml of this solution in another liter of water. The initial pH was adjusted to 8.4 with sodium bicarbonate, and optical sensity readings taken with the Model 450 Colorimeter at different pH points as the pH was lowered with drops of sulfuric acid.

For checking the effect of chlorine upon the dye, a liter of water was adjusted to a pH of 7.7, and 98 ml poured into each of four Nessler tubes. A drop of the stock dye solution containing 328 mg per liter was then placed in each tube. 2 ml of water were then added to the two dye blanks, and 2 ml of 100 ppm of chlorine solution were added to the other two Nessler tubes. Both the dilution water and the chlorine solutions were buffered to pH 7.8. Readings were then taken in the same manner as used above.

The data for both experiments is shown in Tables IIA and IIB, and indicates a loss in optical density caused both by a dropping pH and by chlorine oxidation. It is possible that the dye could have been used at pH values less than 6.8 except for the effect of the chlorine.

6. Comparison of Color Reactions of Chlorine and Chlorine Dioxide Upon Various Proteins and Amino Acids

Early in the work it was found that chlorine dioxide causes visible color changes in solutions of various proteins and amino acids. This was demonstrated by placing 100 ml of buffered water in each of twelve Nessler tubes. Approximately equal portions, or about 20 mg, of the protein or amino acid was then placed in each tube. To each of these tubes was then added 0.5 ml of a buffered 100 ppm chlorine dioxide solution, and the tubes allowed to stand for fifteen minutes.

The same procedure was then repeated with a chlorine solution.

The data are recorded in Table III and show that visible color changes are obtained with chlorine dioxide in the case of the egg and bovine albumin the cysteine, tryptophane, and tyrosine. The chlorine, on the other hand, showed no color change, except that in the case of the tryptophane some color was observed after standing for two to three hours.

7. Comparison of Heat Coagulation of Egg Albumin at pH 4.9 and pH 7.7 Using Optical Density and Turbidity Measurements

In order to have a basis for comparison of heat coagulation with the effect of chlorine and chlorine dioxide it was necessary to actually coagulate some of the egg albumin. It was necessary to use optimum pH conditions for heat coagulation. In order to observe the effect of this coagulation on the optical density and turbidity, measurements were made, before and after centrifuging with both instruments.

A liter of 500 ppm egg albumin was prepared by dissolving 500 mg of the albumin in a liter of water. The pH was adjusted to pH 7.8

with sodium bicarbonate. The solution was pre-centrifuged at 1600 rpm for ten minutes, and then filtered, through glass wool. 200 ml of this solution was then pipetted into each of three beakers, and one of the beakers adjusted to a pH of 4.9, (near the isoelectric point of the protein), with three drops of concentrated acetic acid. Readings were then taken on both colorimeters, (Models 402-E and 450), for the optical density of the samples at both pH values. The procedure for taking turbidity readings on the Model 402-E was as follows:

The instrument was turned on and allowed to warm up for about 15 minutes. The sample holder or cuvette was thoroughly cleaned and washed with de-ionized water. A water blank was used as a standard in setting the machine. The curvette was filled and placed in the sample holder compartment all the way to the right. The reading on the galvanometer was adjusted to zero for the blank with the standardization control knob. Next, the curvette was moved to the other end of the sample holder compartment, i.e., all the way to the left, and the instrument rebalanced with slide wire control. A drop in optical density indicates light scattering or the presence of turbidity. Subsequent readings of the albumin samples were made in the same way.

The reduction of the light on the measuring photocell, brought about by increasing the distance of the sample from the photocell, is due to light being scattered by suspended particles in the sample. The amount of light which strikes the photocell being reduced as the sample is moved away from the photocell is due to turbidity or light scattering effect. This method has the advantage of being sensitive to low turbidity and the readings in this manner are independent of color because, color

does not cause scattering and a suitable monochromatic filter may be used to minimize any color changes that are taking place. A red monochromatic folter, (#M 660), was used in the experiments. The elimination of color was important because chlorine dioxide produces a visible color reaction with the albumin, (as noted previously), and also because the chlorine dioxide has a greenish-yellow color of its own.

The cuvette was thoroughly washed with de-ionized water between each reading, and then washed again with a portion of the sample to be analyzed. It was then refilled and care taken to eliminate the formation of bubbles.

The Model 450 Colorimeter was used for all optical density measurements. 100 ml Nessler tubes were used, each calibrated for a 150 mm light path. This instrument makes it possible to detect very small concentrations of color, and also to note changes in light adsorption due to change in particles size or number of particles. It was necessary to use both colorimeters in order to differentiate between color formation due to reaction of albumin with chlorine dioxide, and any dispersion of coagulation caused by the chlorine dioxide. By using the red, (#650), filter most of the color formed was eliminated, but not all of it. The green, (#530), filter on the other hand, made it possible to eliminate natural green-yellow color of the chlorine dioxide, and allowed for transmission of most of the purplish color formed by the albumin-chlorine dioxide reaction.

In the experiment, two samples, one at pH 4.9, and one at pH 7.8, were heated for ten minutes in a water bath at 80° C. One sample was kept at room temperature. Volume change in the heated samples due to

some evaporation was adjusted with a few drops of buffered water. Readings were then taken on both colorimeters in the manner described above, and taken again after centrifugation.

The data which is recorded in Table IV indicates a number of points which will be used later as a basis of comparison for studying the effect of chlorine and of chlorine dioxide upon the protein. First, it is seen that there is no marked increase in turbidity or optical density of the sample at pH 7.8 upon heating to 80° C. The sample at pH 4.9, on the other hand, shows more than a twofold increase in turbidity upon heating. Upon centrifugation, the sample at pH 7.8 shows a decrease in turbidity of 20 per cent, whereas the sample at pH 4.9 shows a reduction of 80 per cent.

8. Comparison of Chlorine and Chlorine Dioxide Effect Upon Egg Albumin Using Optical Density Measurements

Two liters of 100 ppm egg albumin were prepared and buffered to pH 7.8 with bicarbonate. 180 ml of albumin solution was poured into each of five beakers. The albumin blank was adjusted to a volume of 200 ml with buffered water. Each of the other four beakers were adjusted to strengths of 2, 4, 6, and 8 ppm chlorine, by adding 4, 8, 12, and 16 ml of buffered 100 ppm chlorine solution to each with a pipette. The final volume in each was adjusted to 200 ml by the addition of buffered dilution water. The actual concentration of chlorine then was 2, 4, 6, and 8 ppm, and the actual concentration of albumin was 90 ppm. Samples were then mixed mechanically for twenty minutes in a dark constant-temperature room at 20° C., and readings taken with the Model #450 Colorimeter. The red filter #650 was used. Samples were then centrifuged for ten

minutes at 1600 rpm, allowed to settle, and readings taken.

The data as shown in Table V and Figure 2 indicate an initial drop in optical density with chlorine, and no change in density after centrifugation.

The chlorine dioxide shows a small initial rise in optical density plus a small drop after centrifugation. The final density after centrifugation, however, is still greater than that of the blank.

It is also seen that the rise in density with the chlorine dioxide and the fall with chlorine does not change with increasing increments of the two gases, i.e., a limit of reaction of the gases with the protein has been reached.

9. Comparison of Chlorine and Chlorine Dioxide Effect Upon the Optical Density of Egg Albumin, Using Green Filter #530

The color development of chlorine dioxide and egg albumin as compared to the effect of chlorine is more clearly in evidence when the green (#530) filter is used on the Model #450 Colorimeter. The green filter allows for maximum transmission of the purplish color in the red spectrum produced by the reaction of the chlorine dioxide and at the same time minimizes the natural green-yellow color of the chlorine dioxide.

In this experiment 100 ppm of egg albumin was used with 10 ppm of chlorine and chlorine dioxide.

Samples were diluted to final volume of 200 ml and readings taken. Samples were then re-centrifuged for ten minutes at 1600 rpm and readings taken again.

The data shown in Table VI and Figure 3 indicate again the initial rise in optical density with the chlorine dioxide. The rise is somewhat

higher than with the red filter because more of the color produced is transmitted. Upon centrifugation there is a small drop, but the optical density still remains above that of the blank.

The chlorine samples indicate a small drop in optical density initially and no significant decrease with centrifugation.

10. Effect of Low Concentrations of Chlorine Upon the Turbidity of Solutions of Egg and Bovine Albumin

In order to ascertain whether coagulation could or did occur at concentrations of chlorine below 1 ppm, turbidity analyses were made upon both egg and bovine albumin, using 100 ppm of albumin at pH 7.8.

Turbidity measurements on the Colorimeter Model 402-E were made after twenty minutes of mechanical stirring.

The data recorded in Table VII and the turbidity changes plotted in Figure 4 show no significant change in the turbidity of either the egg or the bovine albumin at the low concentration level studied.

11. Effect of Chlorine Dioxide Upon the Turbidity of Bovine Albumin

Two liters of 500 ppm of bovine albumin were prepared, adjusted to 7.8 and centrifuged for ten minutes at 1600 rpm.

185 ml of the solution was then pipetted into each of six amber colored Erlenmeyer flasks, and increments of 1 to 5 ppm of chlorine dioxide added. Final volume in each flask was adjusted to 200 ml in each case with buffered dilution water.

Solutions were then mixed mechanically for twenty minutes and readings taken with the Colorimeter Model 402-E. Samples were then re-

centrifuged for ten minutes at 1600 rpm, and readings retaken.

The data which are given in Table VIII and the turbidities which are given in Figure 5 again indicate no significant change in the optical density of the protein suspensions either before or after centrifugation.

12. Comparison of Effect of 5 ppm Chlorine and Chlorine Dioxide Upon 500 ppm. of Egg Albumin at pH 7.8, Using Duplicate Samples

In this experiment samples were run in duplicate to check on the reproducibility of the data. Both colorimeters were used in order to measure optical density changes and turbidity. A red filter was used for both instruments.

Two liters of a 500 ppm solution of egg albumin were prepared and buffered to a pH of 7.8. The larger concentration of protein was used in order to extend the sensitivity range of the instruments. The solution was centrifuged for ten minutes at 1600 rpm, and then filtered through fine glass wool to remove the larger particles brought down by centrifuge. 200 ml of this solution was then pipetted into each of six 250 ml amber colored Erlenmeyer flasks. Amber flasks were used here to keep the influence of light on the decomposition of the chlorine and chlorine dioxide at a minimum. 10 ml of a buffered solution of 105 ppm chlorine was then pipetted into each of two flasks, and 10 ml of a buffered solution of 105 ppm chlorine dioxide was pipetted into another two flasks. The last two flasks were used as blanks, and diluted to the same final volume, (210 ml), with buffered dilution water. All samples were then stirred mechanically for twenty minutes. Readings were then taken

on the colorimeter using 150 mm Nessler tubes, and then compared on the other colorimeter using the turbidimetric technique. All samples were then re-centrifuged at 1600 rpm for ten minutes and readings retaken in the same manner.

The data shown in Table IX indicate a small initial drop in optical density with the chlorine with no significant change in density or after centrifugation. The results of the reaction of the albumin with the chlorine dioxide show a small initial rise in optical density and turbidity, as well as an optical density value above that of the blank after centrifugation.

13. Comparison of Effect of Chlorine and Chlorine Dioxide Upon Egg Albumin at pH 7.8 With Readings Taken After Adjustment of pH to Iso-Electric Point (pH 4.6)

Previous mention has been made of the fact that when egg albumin is heated at a pH which is far removed from the iso-electric point the denaturation effect may not be apparent or visible. However, if the pH is lowered back to 4.6 to 4.9, the albumin is seen to coagulate.

The purpose of this experiment was to see if this could be applied to the chlorine and chlorine dioxide effect and to see if any coagulation could be detected after the pH adjustment was made.

A liter of 500 ppm egg albumin at pH 7.8 was prepared and pre-centrifuged at 1600 rpm for ten minutes. It was then filtered through glass wool. 200 ml of solution was then pipetted into each of four beakers. The first two were used as blanks and diluted to 210 ml with buffered dilution water. 10 ml of buffered 105 ppm chlorine was then

added to the third beaker, and 10 ml of 105 ppm chlorine dioxide solution added to the last. Samples were then mixed mechanically for twenty minutes. Beakers containing the chlorine and chlorine dioxide were then checked with orthotolidine to see if any residual, measured as free chlorine, was present in either of the beakers. The residual test was negative, and this eliminated the possibility of any free chlorine or chlorine dioxide reacting with the acetic acid to be added. All samples were adjusted to pH 4.6 with three drops of concentrated acetic acid, and readings taken with both colorimeters, using red filters. The samples were then recentrifuged at 1600 rpm for ten minutes and readings taken again.

The data as shown in Table X indicate a small initial rise in optical density and turbidity with the chlorine, and a somewhat larger rise in density and turbidity with the chlorine dioxide. Upon centrifugation, the optical density and turbidity values of both the chlorine and chlorine dioxide treated samples are still higher than the values of the blank.

CHAPTER IV

DISCUSSION OF RESULTS

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1. Sodium Nitroprusside and Orange I Dye

In the search for an appropriate method for the detection of denaturation of albumin in aqueous solutions, it was thought that since dyes had been used as indicators of denatured albumin by Hopkins and Carroll, that this technique could be used in conjunction with this study.

a. The experiment with the nitroprusside dye indicates the possibility of some coagulation of the albumin after heating, as indicated by a rise in optical density. Because there was no significant change in the hue of color, as shown in Figure 1, (in spite of the increase of the optical density), it was concluded that the dye would make very little contribution to the interpretation of the work of this thesis.

b. The data of the experiment with Orange I dye indicates that both pH and the chlorine itself affect the intensity of the color produced. This would have made interpretation of denaturation on the basis of color change alone almost impossible.

In consideration of the results of these two experiments it was concluded that the application of dyes to this study was impractical, and would not lead to simplification of the technique.

2. Comparison of Color Reactions of Albumins and Amino Acids With Chlorine and Chlorine Dioxide

In view of the difficulties encountered with the dyes, it was thought that a technique utilizing a colorimetric apparatus for the de-

termination of turbidity only might be adequate. It became evident from the first, however, that the technique of interpreting light transmission values for evaluating denaturation would be complicated by the fact that chlorine dioxide has color while chlorine has none; chlorine dioxide rapidly produces a colored product with albumin while chlorine produces this color with albumin very slowly, if at all.

Chlorine dioxide developed a visible color change with both bovine and egg albumin while it failed to develop a color with gelatin. A color change with the amino acids tryptophane and tyrosine is also developed. It has been shown that these amino acids are absent in gelatin.[15] It can be concluded that the color development in albumin with chlorine dioxide is the result of the oxidation of the aromatic ring structures of the organic radicals of tryptophane and tyrosine even though they are present in the peptide chain.

Because the chlorine reaction with the same proteins did not develop color within the time studied, it may be concluded that the chlorine reacts more readily with nitrogen of the peptide chain than with the organic fraction of these acids.

The decolorization of the chlorine dioxide is caused by the oxidation of the sulfhydryl group (SH) of the amino acid cysteine by the gas. The chlorine undoubtedly also attacks this amino acid but this was not demonstrated because of a lack of color change with chlorine.

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[15] M. Sahyun, et al., Outline of the Amino Acids and Proteins, (New York: Reinhold Publishing Corporation, (1948), p. 67.

3. Turbidimetric Analysis of the Chlorine and Chlorine Dioxide Effect Upon Egg and Bovine Albumin

The color complication caused by the reaction of chlorine dioxide with albumin necessitated the use of a means of measuring light scattering with another instrument described in the previous chapter, in conjunction with a procedure using purely light adsorption. The turbidimetric, (light scattering), technique made it possible to separate the rise in optical density caused by color formation from a rise in optical density caused by coagulation or dispersion. This would not have been necessary in the case of the chlorine since it appeared to develop no color within the time studied.

The results of the turbidity and light adsorption studies carried on in Experiments 7 through 13, and the data of which are shown in Tables IV to X, and Figures 2 to 5, indicate the following:

a. In the heat coagulation of albumin, the pH is a critical factor. There was no appreciable rise in optical density, and hence no marked coagulation at a pH of 7.8. The turbidity of the sample at pH 4.9 was more than doubled at the same temperature and within the time allowed for the reaction. With centrifugation, the samples at pH 7.8 showed a decrease in turbidity of 20 per cent. The sample at pH 4.9, on the other hand, showed a decrease of 80 per cent. As a basis of comparison, then, if the chlorine or chlorine dioxide were to show the effect of coagulation of the albumin, there would first have to be a marked rise in optical density and turbidity, followed by a definite decrease in optical density and turbidity after centrifugation.

b. The experiments which followed, however, indicated that the

effect of chlorine and chlorine dioxide on the egg and bovine albumin does not follow the pattern described above. In the case of the chlorine dioxide, light adsorption studies with the egg albumin indicated an initial rise in optical density over and above that of the blank, and that this rise persisted after centrifugation. It was shown that this optical density rise is partly due to the color formation developed by reaction of the gas with the aromatic acid radicals of the albumin. The light adsorption analysis of the chlorine dioxide- egg albumin reaction, using a red filter which minimized the color produced, further indicated that the effect of the gas reached a maximum, i.e., there was no further adsorption of the gas on the egg albumin molecule with additional increments of chlorine dioxide. The turbidimetric analysis of the same reaction of chlorine dioxide with egg albumin indicated that this rise in optical density was also caused by some dispersion of the particles in solution, since the initial turbidity was higher than that of the blank. Since neither the optical density or the turbidity of the egg albumin dropped below that of the blank after centrifugation, it was concluded that coagulation had not occurred. This was further confirmed by the last experiment in which a pH adjustment to 4.6 was made after allowing the initial reaction to proceed at pH 7.8. Here again the results showed no reduction in optical density and turbidity after centrifugation, below that of the blank, which was also adjusted in pH and centrifuged.

The reaction of the chlorine dioxide with the bovine albumin confirmed the color formation, but showed no significant change in turbidity.

c. The analysis as applied to the reaction of chlorine with egg and bovine albumin, again indicated that coagulation did not occur. The light adsorption studies of chlorine and egg albumin, as shown in Figures 2 and 3, indicated an initial drop in optical density, and no significant change upon centrifugation. The drop in optical density, as shown in Figure 2 reached a maximum and did not decrease further with further addition of the chlorine. Apparently, then, a point of maximum adsorption of chlorine on the egg albumin had been reached. The turbidimetric analysis of the reaction of chlorine with egg albumin indicated that there was a small decrease in turbidity initially, but that there was no significant difference from the blank after centrifugation. It would be difficult to explain the initial drop in optical density and turbidity of egg albumin caused by the chlorine except on the basis of some slight change in particle size. Possible it is due to some shrinkage in size of the particles, or some cohesion due to polarity changes on the particles.

The effect of the chlorine upon the bovine albumin indicated no significant changes in turbidity.

In conclusion, it is evident from the results of the above experiments, using both the light adsorption and turbidimetric techniques, that coagulation of both the egg and bovine albumin is not brought about by the reaction of either of the two gases, at the pH and concentration levels used.

In keeping with the various theories for denaturation discussed earlier, this would mean that there apparently is no rupture of the salt bridge due to the attack of the amine radical of the lysine and

arginine acid fractions by either of the two gases. If we think of denaturation as being due to the unfolding of the closely packed peptide chains, then, here too, it would seem that there is no primary attack at the point of folding, since coagulation does not occur.

The color produced by the chlorine dioxide with egg and bovine albumin indicates attack of the tryptophane and tryosine amino acid organic fractions. The change in solubility of the organic fraction of these acids may account for the apparent dispersion of egg albumin by this gas.

The chlorine was in general found to be less reactive, and this was surprising in view of the fact that chlorine has been stated to have the property of coagulating and precipitating certain proteins in waste from slaughter houses,[16] but that this occurs only when high, (50 ppm), chlorine residuals are used.[17] The ratio of the chlorine to the protein was not indicated in the article. On the other hand, the effect of chlorine in bacterial kill is felt even at the trace level, (0.2 to 2.0 ppm).

The apparent greater reactivity of chlorine dioxide with bacteria and in developing color in albumin as compared to chlorine may be partially explained on the basis of a more rapid adsorption of this gas on the large albumin molecules and the bacteria. The work of

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[16] E. F. Eldridge, Industrial Waste Treatment Practice, (New York: McGraw Hill Book Company, Incorporated, 1942), p. 272.

[17] Ibid., p. 273.

Ingols and Ridenour[18] has shown that chlorine dioxide reacts with the large molecule of peptone in an amount which follows the laws of adsorption on the molecule. The same writers[19] have also shown that the relative effectiveness of chlorine dioxide as a bactericide is increased as the concentration of other colloidal substances which might compete for the gas is decreased.

This difference in the rate of adsorption, and in the nature of the chemical reactivity discussed earlier, rather than any difference attributed to coagulating properties which have been shown to be absent, may account for the difference in the bactericidal effect of the two gases. It has been shown that chlorine dioxide is more effective than chlorine in killing cultures of poliomyelitis virus and E. coli.[20]

The matter of the oxidation of the amino acid radicals by the chlorine dioxide and its possible effect on the cellular activity or the protoplasmic entity, has as yet not been investigated, and therefore no conclusions can be drawn until some experimental evidence is available.

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[18] R. S. Ingols and G. M. Ridenour, Jour. A. W. W. A., 40, 1224 (1948).

[19] Ibid., p. 1224.

[20] Ibid., p. 1223.

CHAPTER V

SUMMARY

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SUMMARY

1. The use of the two dyes, sodium-nitroprusside and Orange I were not found to be of practical use for the detection of denaturation in aqueous solutions of albumin when using chlorine and chlorine dioxide as the possible denaturants.

2. In the analysis of the effect of the two gases upon egg and bovine albumin by light adsorption, it was found that chlorine dioxide has properties differing from those of chlorine. Chlorine dioxide develops color with both egg and bovine albumin, and with the aromatic amino acids, tyrosine and tyrptophane which are present in albumin. This is presumed to be caused by the oxidation of the aromatic rings of these acids, and apparently also results in some dispersion of the egg albumin. Chlorine, by contrast, developed no color with the albumins during the time studied but did show some effect on the egg albumin particles. The effect observed was apparently the result of some shrinkage, or cohesion of the individual egg albumin particles by the chlorine. This change in particle size by both gases evidently reaches a maximum at the maximum adsorption level, and does not show further change with increasing dosages of the gases.

3. Using the technique of light adsorption and of turbidimetry (light scattering) followed by centrifugation, and using the heat coagulation of egg albumin as a basis of comparison, it was found that neither the chlorine or the chlorine dioxide follows a pattern which is similar to

typical coagulation phenomena. Adjustment of the pH to the iso-electric point after reaction period between the gas and the protein, confirmed the fact that no coagulation had taken place.

4. Some of the qualitative changes observed in the effects of the two gases, i.e., the color changes and some change in particle size, may be a factor in the bactericidal properties of the two gases. An exact interpretation of this phenomenon in terms of the effect of the gases on the enzymic activity of the bacterial cell, is not possible until some experimental data become available.

CHAPTER VI

SUGGESTIONS FOR FURTHER STUDY

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1. It is realized that the problem of protein denaturation by chlorine and chlorine dioxide has not been entirely solved or exhausted. A study using a wider range of pH and a wider range of concentration of albumin and chlorine and chlorine dioxide is indicated. It is possible that coagulation may occur at a critical concentration of reactants, and optimum pH conditions.

2. The technique of analyzing the degree of denaturation or coagulation may not be the best one. It may be possible to develop other techniques for studies at the smaller protein concentration levels.

3. The effect of chlorine, and of chlorine dioxide in particular, upon the various amino acids should be further investigated. The color produced by the chlorine dioxide cannot be interpreted properly until we have studied the possible correlation of the changes with enzymic activity.

4. The albumins used in the investigation may not be truly analogous to the protoplasm of bacterial cells. It is suggested, therefore, that similar studies be made using other types of proteins, or mixtures of proteins.

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APPENDIX I

TABLES

TABLE I

TEMPERATURE EFFECT UPON 100 ppm EGG ALBUMIN, pH 7.8 WITH SODIUM-NITROPRUSSIDE DYE, USING ALL COLOR FILTERS, COLORIMETER MODEL 450*

	Optical Densities				
Temperature °C**	Filter #390	Filter #490	Filter #530	Filter #580	Filter #650
23	1.65	1.55	1.10	.65	.35
40	1.55	1.45	1.00	.60	.30
60	1.75	1.75	1.20	.70	.70
80	2.00	2.00	1.45	.95	.55

*Read at room temperature.
**Temperature to which sample was heated.

TABLE IIA

EFFECT OF pH UPON THE OPTICAL DENSITY OF ORANGE I DYE, READINGS WITH COLORIMETER MODEL 450, GREEN FILTER #530

pH	Optical Density
8.4	3.5
8.0	3.2
6.9	2.8
6.8	2.7
5.6	2.7
4.0	2.7
2.8	2.7

TABLE IIB

EFFECT OF CHLORINE UPON THE OPTICAL DENSITY OF ORANGE I DYE, READINGS WITH COLORIMETER MODEL 450, GREEN FILTER #530

ppm Cl_2	Optical Density
0	0.25
0	0.25
2	0.15
2	0.15

TABLE III

COMPARISON OF COLOR REACTIONS* OF CHLORINE DIOXIDE AND OF CHLORINE UPON VARIOUS PROTEINS AND AMINO ACIDS

Protein or Amino Acid	Color Change With ClO_2	Color Change With Cl_2
Egg Albumin	+	-
Bovine Albumin	+	-
Gelatin	-	-
Cystine	-	-
Cysteine	Rapid Decolorization**	-
Valine	-	-
Arginine	-	-
Lysine	-	-
Tryptophane	+	-***
Histidine	-	-
Proline	-	-
Tyrosine	+	-

*At the end of 15 to 30 minutes.
**Reduction of the ClO_2 and oxidation of cysteine to cystine.
***Slight color developed after 2-3 hours.

TABLE IV

COMPARISON OF HEAT COAGULATION OF 500 ppm EGG ALBUMIN AT pH 4.9 AND pH 7.8, USING COLORIMETERS 450 AND 402-E, RED FILTERS #650 AND #M-660

Temperature °C*	pH	Before Centrifuge Optical Density	Before Centrifuge Turbidity	After Centrifuge Optical Density	After Centrifuge Turbidity	Per Cent Reduction in Turbidity
25	7.8	1.75	10.5	-	-	-
25	4.9	1.75	10.7	-	-	-
80	7.8	1.80	10.6	1.35	8.2	20
80	4.9	7.20	23.0	.85	5.0	80

*Read at room temperature.

TABLE V

COMPARISON OF CHLORINE AND CHLORINE DIOXIDE EFFECT UPON EGG ALBUMIN (90 ppm) pH 7.8, WITH COLORIMETER #450, RED FILTER #650

Concentration Chlorine ppm	Concentration Chlorine Dioxide ppm	Optical Density Before Centrifuge	Optical Density After Centrifuge
0	0	.50	.35
2	0	.30	.30
4	0	.30	.30
6	0	.30	.30
8	0	.30	.30
0	2	.55	.50
0	4	.55	.50
0	6	.55	.50
0	8	.55	.50

TABLE VI

COMPARISON OF EFFECT OF CHLORINE AND OF CHLORINE DIOXIDE UPON EGG ALBUMIN, 100 ppm AT pH 7.8 GREEN FILTER #530 WITH COLORIMETER MODEL 450

Sample V	Optical Density Before Centrifuge	Optical Density After Centrifuge
Albumin Blank	.85	.60
Albumin + 10 ppm Chlorine	.50	.50
Albumin + 10 ppm Chlorine Dioxide	1.70	1.55

TABLE VII

EFFECT OF LOW CONCENTRATIONS OF CHLORINE UPON THE TURBIDITY OF EGG AND BOVINE ALBUMIN, 100 ppm, pH 7.8, COLORIMETER MODEL #402-E, RED FILTER #M-660

Sample	Chlorine ppm	Turbidity
Egg Albumin	0	4.8
Egg Albumin	.2	4.4
Egg Albumin	.4	4.3
Egg Albumin	.8	4.6
Bovine Albumin	0	2.2
Bovine Albumin	.2	1.9
Bovine Albumin	.4	2.0
Bovine Albumin	.8	2.1

TABLE VIII

EFFECT OF CHLORINE DIOXIDE UPON THE TURBIDITY OF BOVINE ALBUMIN, pH 7.8, 500 ppm, pH 7.8, WITH COLORIMETER MODEL 402-E, RED FILTER #M-660

Concentration Chlorine Dioxide ppm	Turbidity Before Centrifuge	Turbidity After Centrifuge
0	2.0	2.0
1	2.0	2.0
2	1.9	2.0
3	2.0	2.0
4	1.9	2.0
5	2.0	2.0

TABLE IX

EFFECT OF 5 ppm CHLORINE AND CHLORINE DIOXIDE UPON THE OPTICAL DENSITY AND TURBIDITY OF 500 ppm EGG ALBUMIN, pH 7.8, USING COLORIMETERS MODELS 450 AND 402-E, RED FILTERS #650 AND #M-660, DUPLICATE SAMPLES

Concentration of Chlorine or Chlorine Dioxide	Before Centrifuge		After Centrifuge	
	Optical Density	Turbidity	Optical Density	Turbidity
0	1.6	10.2	1.0	6.6
0	1.6	10.0	1.0	6.6
5 ppm Cl_2	1.4	9.0	1.0	6.5
5 ppm Cl_2	1.4	9.0	1.0	6.4
5 ppm ClO_2	2.1	10.7	1.9	9.5
5 ppm ClO_2	2.1	10.7	1.8	9.5

TABLE X

COMPARISON OF EFFECT OF CHLORINE AND CHLORINE DIOXIDE UPON 500 ppm EGG ALBUMIN AT pH 7.8, WITH READINGS TAKEN AFTER ADJUSTMENT OF pH TO 4.6, COLORIMETER MODELS 450 AND 402-E, RED FILTERS #650 AND #M-660

Concentration of Chlorine or Chlorine Dioxide	Before Centrifuge Optical Density	Before Centrifuge Turbidity	After Centrifuge Optical Density	After Centrifuge Turbidity
0	1.25	7.80	.85	6.1
0	1.25	7.80	.90	6.2
5 ppm Cl_2	1.35	8.00	1.00	6.5
5 ppm ClO_2	2.20	10.90	1.65	7.9

APPENDIX II

FIGURES

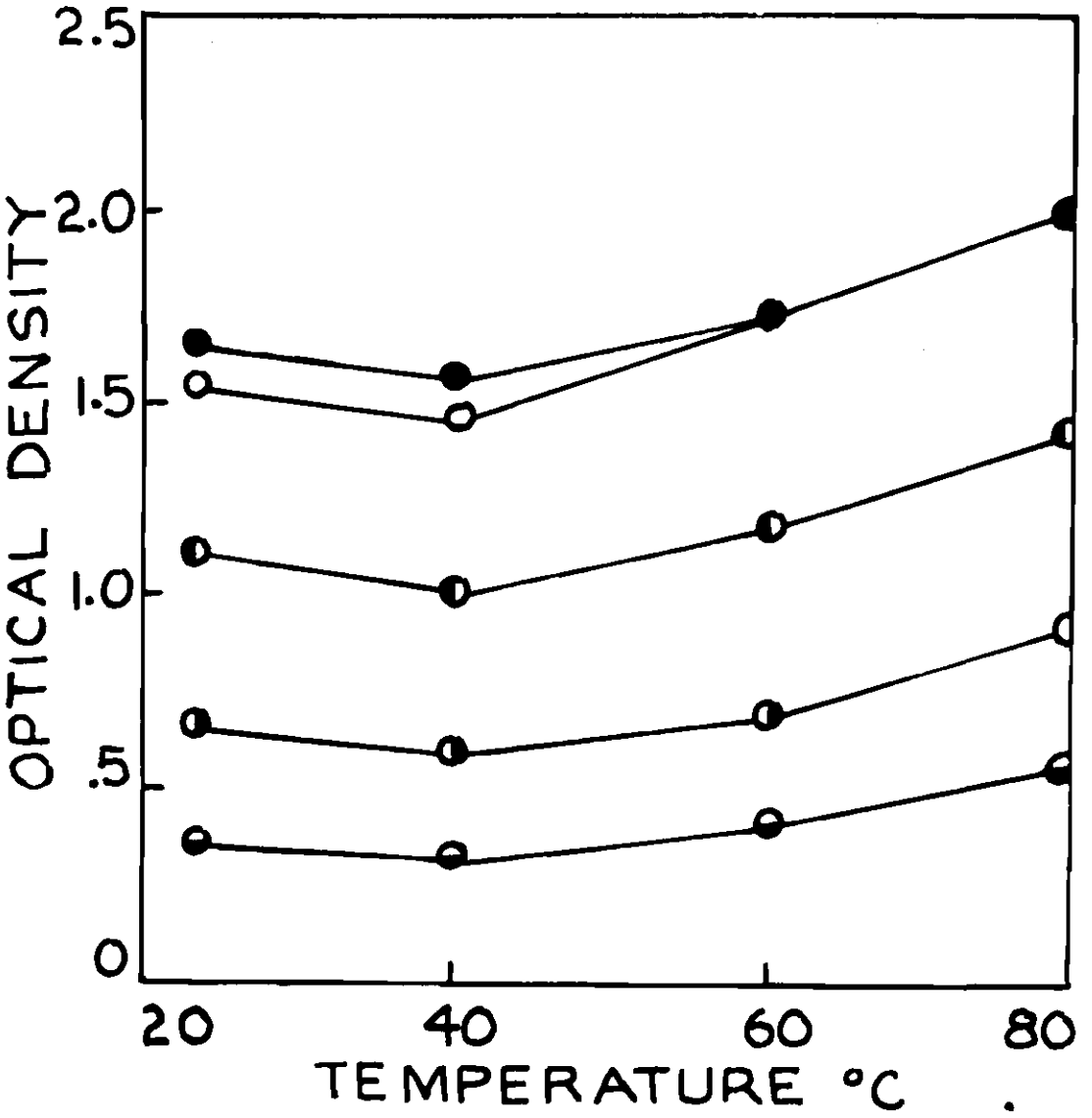

FIGURE 1

TEMPERATURE-OPTICAL DENSITY DIAGRAM OF NITROPRUSSIDE DYE AND 100 ppm EGG ALBUMIN pH 7.8, COLORIMETER MODEL 450 COLOR FILTERS #390 ●, #490 ○, #530 ◐, #580 ◑, AND #650 ◒

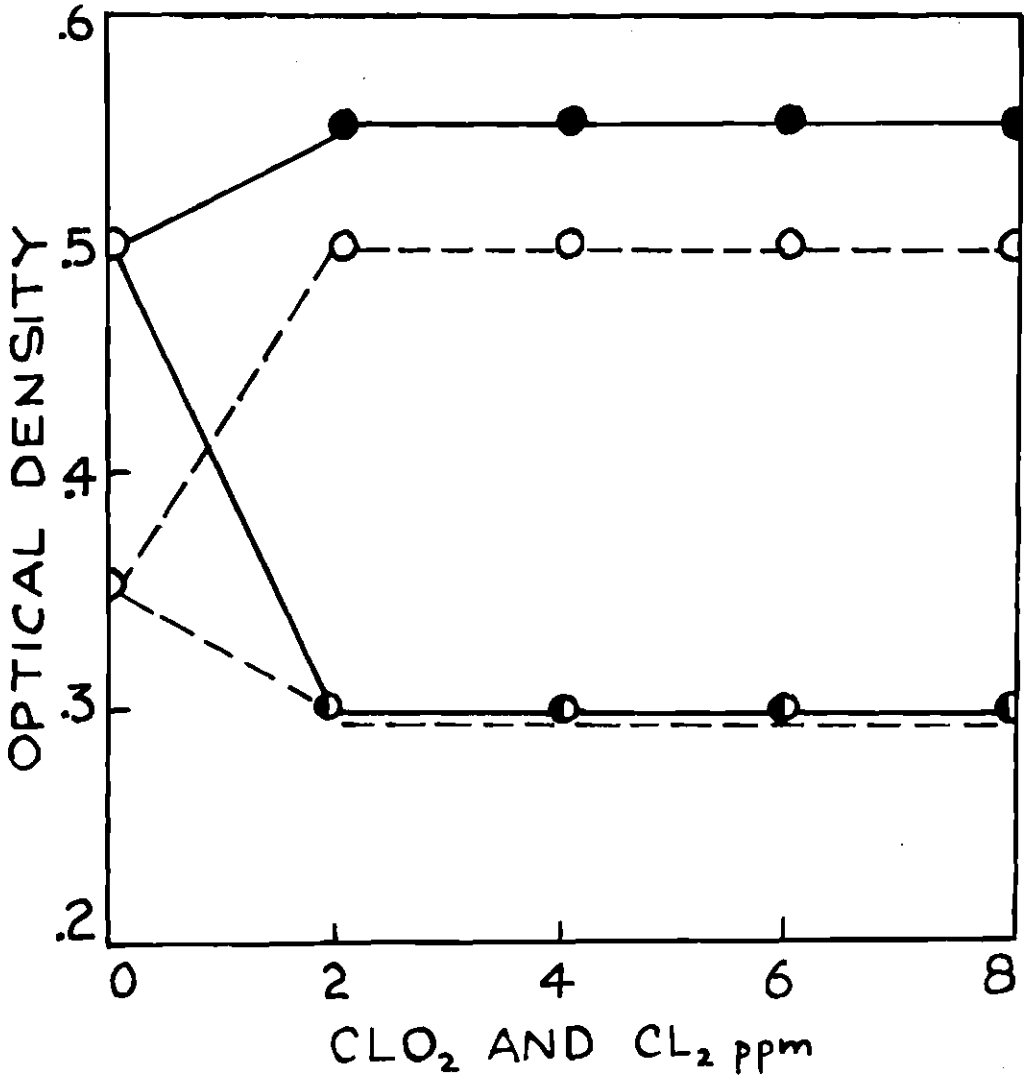

FIGURE 2

OPTICAL DENSITY DIAGRAM OF CHLORINE DIOXIDE AND CHLORINE WITH 90 ppm EGG ALBUMIN, pH 7.8, COLORIMETER MODEL 450, RED FILTER #650

LEGEND:
- ALBUMIN + CLO_2
- ALBUMIN + CLO_2 + CENTRIFUGE
- ALBUMIN + CL_2
- ALBUMIN + CL_2 + CENTRIFUGE

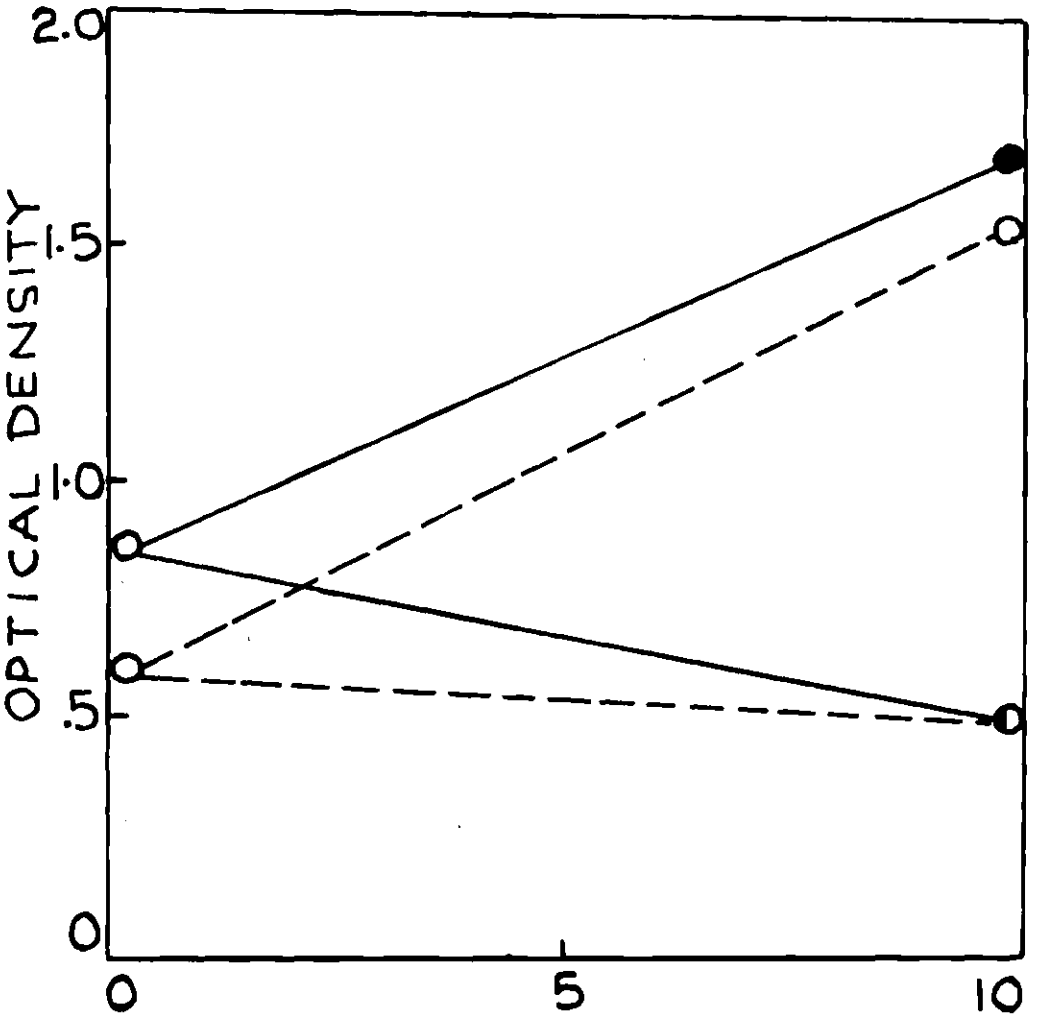

FIGURE 3

OPTICAL DENSITY DIAGRAM OF CHLORINE DIOXIDE AND CHLORINE WITH 100ppm EGG ALBUMIN, pH 7.8 COLORIMETER MODEL 450, GREEN FILTER #530

LEGEND: O—● ALBUMIN + CLO_2

O--O ALBUMIN + CLO_2 + CENTRIFUGE

O—◐ ALBUMIN + CL_2

O--◐ ALBUMIN + CL_2 + CENTRIFUGE

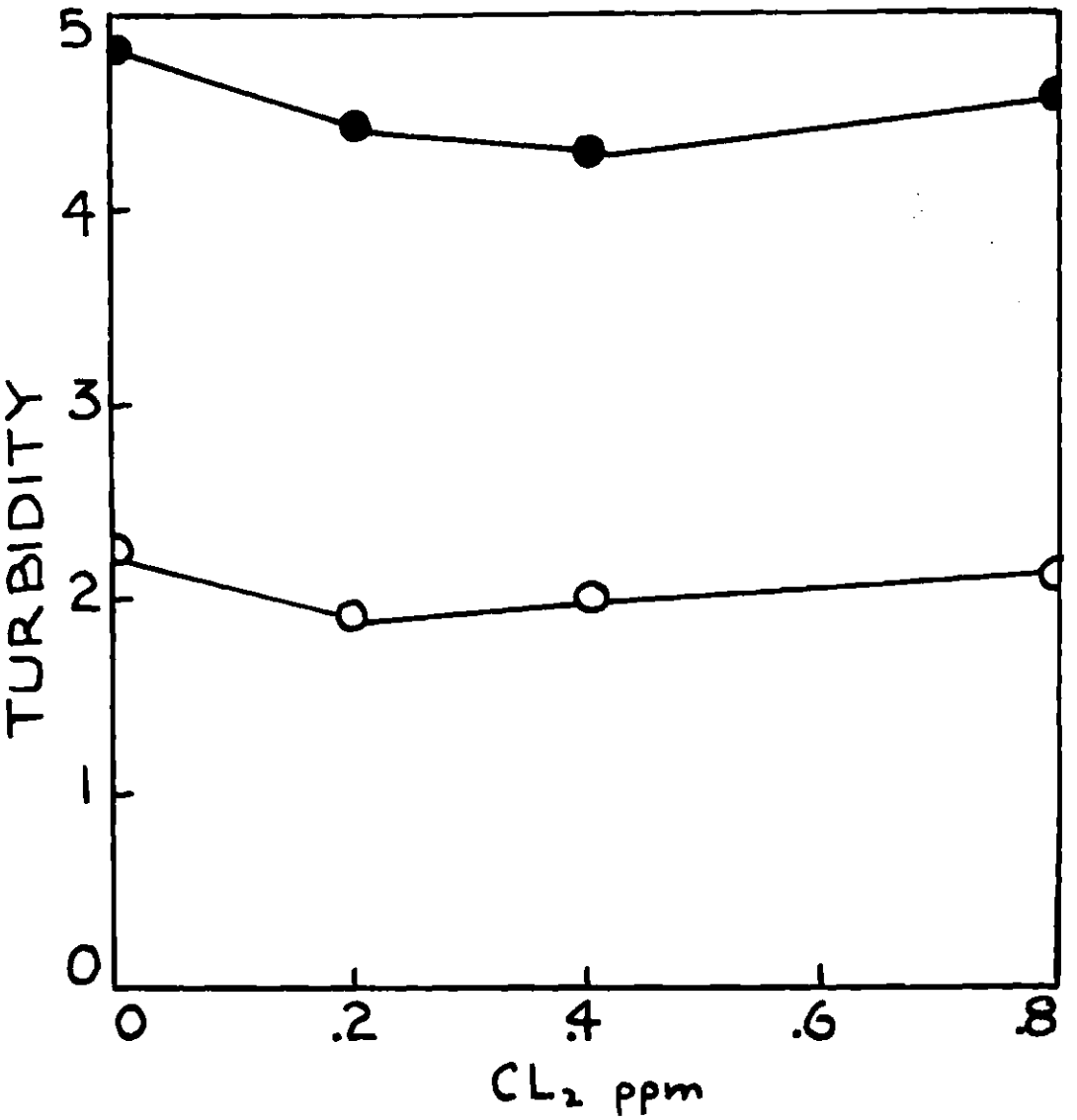

FIGURE 4

TURBIDITY DIAGRAM OF LOW CHLORINE CONCENTRATION WITH 100 ppm EGG AND BOVINE ALBUMIN pH 7.8, COLORIMETER MODEL 402-E, RED FILTER #M-660

LEGEND: ●—● CL_2 + EGG ALBUMIN

O—O CL_2 + BOVINE ALBUMIN

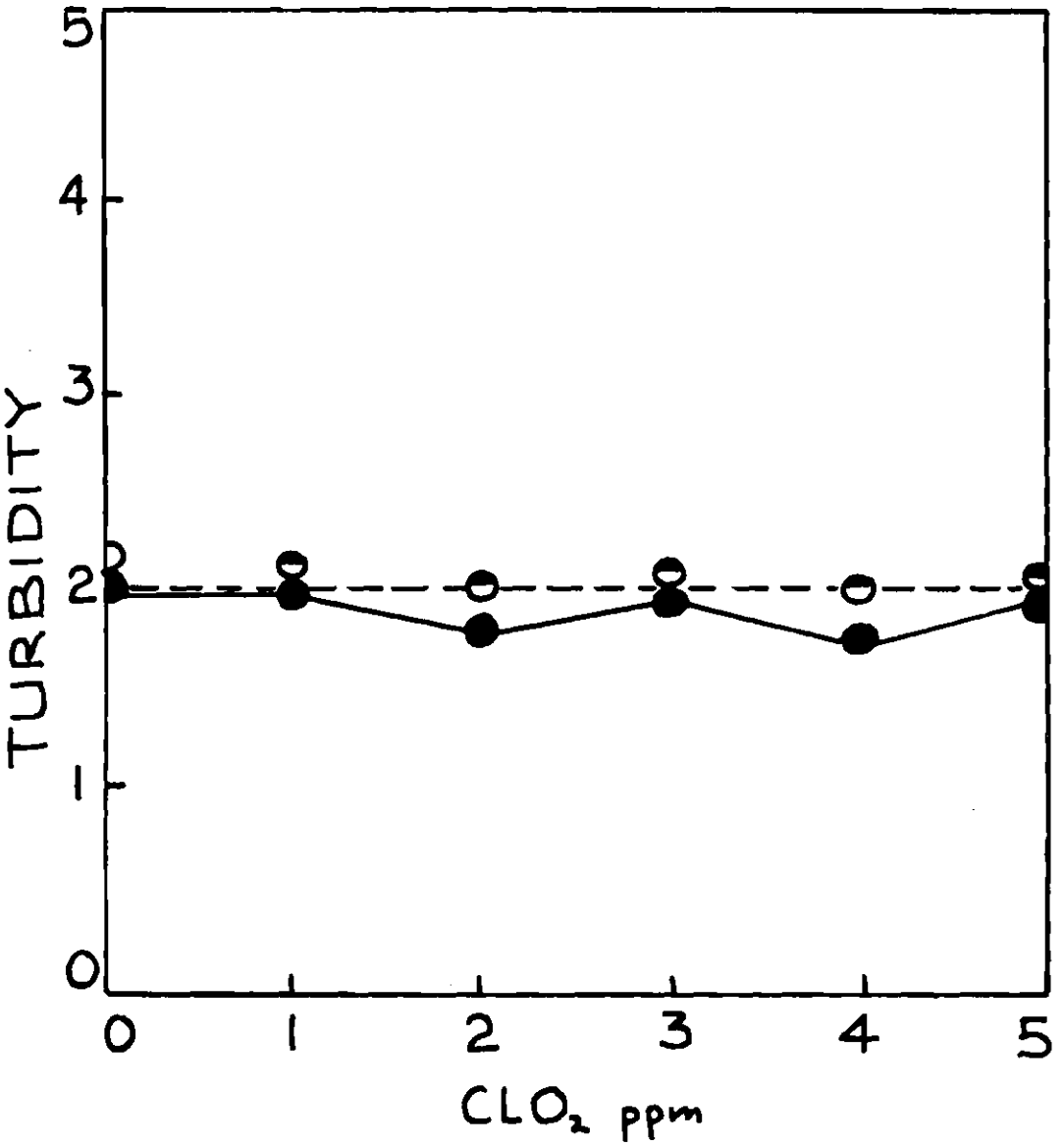

FIGURE 5

TURBIDITY DIAGRAM OF CHLORINE DIOXIDE WITH 500ppm BOVINE ALBUMIN, pH 7.8, COLORIMETER 402-E, RED FILTER # M-660

LEGEND : ●—● ALBUMIN + CLO_2

○—○ ALBUMIN + CLO_2 + CENTRIFUGE